

Short communication

Characterization of a factor from bovine intestine that protects against *Cryptosporidium parvum* infection

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Abstract

Cryptosporidium parvum is a protozoan parasite that causes intestinal infection in a variety of mammals. We have previously described a factor in adult rat or adult bovine intestinal mucosa that protects against *C. parvum* infection when fed to susceptible infant rats. This factor is absent in intestinal mucosa from bovine calves. In the present study we describe the further characterization of the active component of bovine intestinal mucosa. The ability to protect infant rats against *C. parvum* infection was found to be associated with the extrinsic membrane protein fraction of the intestinal mucosa. Extrinsic membrane preparations from adult cows, adult rats, and calves were separated by SDS-PAGE. A band with apparent molecular mass of 54 kDa was seen in preparations from adult rat and cow, but not calf. Protein was transferred to PVDF membrane and from this the band was excised and subjected to N-terminal sequence analysis using a gas-phase protein sequenator. A 15-amino acid consensus sequence was generated with homology to leucine aminopeptidase (LAP). Purified LAP was purchased from a commercial source and tested for ability to protect infant rats against *C. parvum* infection. Rats fed LAP from 7 to 11 days of age and challenged with *C. parvum* at 9 days were significantly less infected than controls upon necropsy at 15 days of age. These data suggest that a protein with N-terminal sequence homology to LAP may reduce susceptibility of infant rats to *C. parvum* infection.

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1. Introduction

Cryptosporidium parvum is an intracellular protozoan parasite that causes intestinal infection and diarrhea in a wide variety of mammalian hosts (Casemore et al., 1997; O'Donoghue, 1995). In spite of intensive research, no effective drugs are available to treat this disease in animals or humans (Blagburn and

Soave, 1997; Harp, 2003). In addition, although some vaccines have proven partially effective in experimental trials, none have been shown to be effective under field conditions (Harp and Goff, 1995; Harp et al., 1996; Perryman et al., 1999). Young animals are generally more susceptible to infection with *C. parvum* than are adults (Angus, 1990; Ungar, 1990). In rodents, infants are susceptible up until the age of weaning, while adults are resistant even without prior exposure to the disease (Ernest et al., 1986; Harp et al., 1988; Sherwood et al., 1982). This suggests that some physiologic change in the intestine, rather than acquired immunity, may be at least partly responsible for the resistance of adults to

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infection. Johnson et al. (2004) isolated a lipid moiety from bovine intestine that inhibited binding of *C. parvum* sporozoites to MDBK cells in vitro. In previous studies, we found that oral inoculation with intestinal mucosa of adult rats or cows was able to protect infant rats from *C. parvum* infection (Akili and Harp, 2000; Harp, 2003). Mucosa from calves that had recovered from *C. parvum* infection was not able to transfer protection. These data suggested that the ability of the mucosa to protect against infection depended more on the degree of intestinal maturation of the donor, rather than a specific immune status relative to *C. parvum*. In the present study, we report the further characterization of the active component from intestinal mucosa that provides partial protection against *C. parvum* infection when fed to infant rats.

2. Materials and methods

2.1. Study design

In the first study, pooled intestinal mucosa from the four cattle described below was separated into cytosol and membrane fractions. These fractions were then tested for in vivo activity against *C. parvum* (experiment 1). The membrane fraction was further separated into intrinsic and extrinsic fractions, and tested for in vivo activity (experiment 2). The extrinsic fraction was then digested with proteinase K to determine if this would affect in vivo activity against *C. parvum* (experiment 3). In the second study, extrinsic fractions prepared from the cattle, calf, and rat pools described below were separated by SDS-PAGE and a protein band found in cattle and rat preparations [protective against *C. parvum* (this study, and Akili and Harp, 2000)], but not calf preparations [not protective (Akili and Harp, 2000)] was excised and subjected to N-terminal sequencing. In the third study, leucine aminopeptidase (LAP), which had high N-terminal homology with the protein identified by SDS-PAGE, was tested for in vivo activity against *C. parvum*.

2.2. Preparation of intestinal mucosal fractions

Intestinal mucosa was collected from four (one Jersey and three Hereford) female bovines 8–24 months old, and pooled (designated “cow”). A second pool was prepared from intestinal mucosa from two 3 months old Jersey calves that had been previously infected with, and recovered from, *C. parvum* (designated “calf”). A third pool was prepared from intestinal mucosa from Harlan Sprague–Dawley adult rats. These procedures

have been previously described in detail (Akili and Harp, 2000). Preparation of fractions was as follows: Homogenized intestinal mucosa was centrifuged at $100,000 \times g$ for 60 min at 4 °C. The supernatant was designated “crude cytosol” and the pellet was designated “crude membrane”. Crude membranes were pooled and resuspended in PBS. For separation into extrinsic and intrinsic membrane preparations, crude membrane suspension was centrifuged at $100,000 \times g$ for 60 min at 4 °C. The supernatant fluid was discarded and the crude membrane pellets were resuspended in 0.3 M sucrose with 10 mM Tris–HCl, pH 7.5. The resuspended pellets were mixed with 0.1 M sodium carbonate solution at pH 11.5. The solutions were incubated at 4 °C for 30 min, and centrifuged at $100,000 \times g$ for 60 min at 4 °C. The supernatant (containing the extrinsic membrane fraction) was adjusted to pH 7.5–8.0 with HCl, and placed in a 350 ml stirred cell (Amicon, Danvers, Massachusetts) to undergo ultrafiltration pressure dialysis. The retentate was dialyzed and then washed with the same volume of PBS to remove the sodium carbonate. The resultant extrinsic membrane preparation was diluted to the original volume, and stored at –20 °C until tested for biological activity in infant rats. The pellets from the sucrose/sodium carbonate centrifugation step (containing the intrinsic membrane fraction) were diluted in their original volume of PBS, pooled, and resuspended. The resultant intrinsic membrane preparation was stored at –20 °C until tested for biological activity in infant rats. Proteinase K (Sigma Chemical Company, St. Louis, MO) was used to digest proteins in the extrinsic fraction. Proteinase K was made up at a concentration of 20 mg/ml in PBS. It was determined in preliminary studies that incubating proteinase K with the extrinsic protein fraction (4 mg/ml protein) for 24 h at room temperature at a ratio of 1:10 was sufficient to completely digest the proteins in the preparation as determined by the absence of bands on SDS-polyacrylamide gel electrophoresis (PAGE).

2.3. Bioassay of mucosal fractions

Harlan Sprague–Dawley rats (HSD, Madison, Wisconsin) were maintained and bred at the National Animal Disease Center (NADC), Ames, Iowa. The rats were given free access to food and water and were housed in a room with alternating 12 h periods of light and darkness. (All procedures involving animals were approved by the NADC Animal Care and Use Committee.) Intestinal mucosa preparations were fed orally to infant rats starting at 7 days of age up to 11

days of age. Rats received twice daily doses of 200 μ l on day 7, 250 μ l on days 8, 9, 10, and 400 μ l on day 11. At 9 days of age, animals in both the control and treatment groups received an oral inoculation consisting of *C. parvum* oocysts suspended in PBS. The number of oocysts received varied between experiments (1.5×10^2 , or 10^3); however, within each experiment control and treatment groups received the same number of oocysts. The number of oocysts used was based on the 50% infectious dose (ID_{50}) of the oocyst pool for infant mice (Harp et al., 1988). The pups in the treatment groups received the dose of oocysts 0.5 h following treatment with intestinal mucosa preparations. For testing the effects of commercially obtained LAP, rat pups were dosed twice daily from days 7 to 11 with 0.48 mg of LAP in 200 μ l of PBS, and inoculated with 10^3 *C. parvum* oocysts at 9 days. All pups were killed on day 15 of age. Sections of the ileum, cecum, and colon were removed, placed in tissue cassettes, fixed using Z-fix (buffered aqueous zinc formalin, Anatech Ltd., Battle Creek, MI) and embedded in paraffin. Thin sections (3–4 μ m thick) were prepared on slides and then stained with hematoxylin and eosin. The sections were then examined for *C. parvum* using light microscopy. Each slide was scored using an arbitrary scale from 0 to 2 indicating the intensity of infection in the tissue section. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Colon content was also taken from the distal colon. The samples from each of the animals were smeared on a glass slide and stained with carbol fuchsin. Slides were examined using oil immersion (500 \times) under light microscopy to determine the presence or absence of *C. parvum* oocysts.

2.4. Characterization of proteins

SDS-PAGE was used to separate and compare extrinsic membrane preparations. Bis-Tris NuPAGE gels (4–12%) with MOPS running buffer (NOVEX, San Diego, CA) were run according to manufacturer's instructions to visualize protein bands. Molecular weight standard used was SeeBlue prestained standard (NOVEX). Protein was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The gels were loaded with 40 μ g of protein per lane. The gels were stained using Coomassie Blue/GelCode Blue Stain Reagent (Pierce, Rockford, IL) or Silver Stain Plus Kit (Bio-Rad). Proteins were then transferred by electroblotting to polyvinylidene fluoride (PVDF) membranes. Individual bands of interest were excised from the PVDF membranes and subjected to N-terminal sequen-

cing using a Model 494 Procise HT gas phase protein sequenator (Applied Biosystems, Foster City, CA).

2.5. Statistical analysis

Differences between the numbers of animals infected in control versus each of the treatment groups were analyzed using Fisher's exact test. Differences in infectivity scores of treated and control animals were compared using Student's *t*-test. Significance level was set at $P < 0.05$.

3. Results and discussion

Table 1 presents results of three experiments, each with its own set of control animals to account for any potential differences over time and among different batches of rats. We began with separation by centrifugation into a crude membrane and crude cytosol fraction. The activity was clearly present in the crude membrane, and

Table 1

Effect of fractions from pooled female bovine intestinal mucosa on susceptibility of rat pups to *C. parvum* infection^a

Experiment/treatment	Number of animals infected ^b /number tested
Experiment 1	
Control	22/24
Whole scrapings	12/20*
Crude cytosol	27/30
Crude membrane	18/30**
Experiment 2	
Control	27/34
Crude membrane	6/20***
Intrinsic fraction	12/30**
Extrinsic fraction	3/29***
Experiment 3	
Control	31/35
Extrinsic fraction	14/30***
Extrinsic + proteinase K	23/30

^a All animals in experiments 1 and 2 were orally challenged with 10^3 *C. parvum* oocysts at 9 days of age. All animals in experiment 3 were orally challenged with 1.5×10^2 *C. parvum* oocysts at 9 days of age.

^b In experiment 1, number of infected animals was determined by examination of both colon content and intestinal tissue following euthanasia at 15 days of age. Finding *C. parvum* by either method was sufficient to categorize the animal as positive. In experiments 2 and 3, number of infected animals was determined by examination of colon content as this was found to be the more sensitive method.

* Significantly ($P < 0.05$) fewer animals infected than in control group.

** Significantly ($P < 0.01$) fewer animals infected than in control group.

*** Significantly ($P < 0.001$) fewer animals infected than in control group.

not the crude cytosol fraction (Table 1, experiment 1). We then separated the intrinsic, tightly bound membrane components, from the extrinsic, loosely bound components. As shown in Table 1 (experiment 2), the extrinsic membrane fraction clearly was most protective against *C. parvum* infection in the infant rats. To confirm our suspicion that the active component was a protein, we subjected the extrinsic membrane fraction to treatment with proteinase K. This treatment abrogated the protective effect of the preparation, thus indicating that the active component was a protein, or depended on protein for activity (Table 1, experiment 3). To further characterize the active component, extrinsic membranes were prepared from mucosa of adult rats and cows (previously shown to protect infant rats from *C. parvum* infection) and calves (shown to lack protective effect). These were run on SDS-PAGE to separate and compare protein bands between the three preparations. As shown in Fig. 1, a band with apparent molecular mass of 54 kDa was present in preparations from cows and adult rats, but not from calves. Extrinsic membrane preparation from cow mucosa was then run on SDS-PAGE and electro-blotted to PVDF membrane. The 54 kDa band was excised and the first 15 N-terminal amino acids sequenced. The consensus sequence generated had high homology to LAP. Nine of the 15 amino acids matched exactly with known sequence of bovine LAP, and 10 of 15 matched exactly with sequences of human and porcine LAP (data not shown). We then tested commercially prepared porcine LAP (Sigma) for the ability to protect infant rats from infection with *C. parvum*. Rats receiving LAP were less infected than were controls. As shown in

Table 2

Reduction of *C. parvum* infection in rat pups following treatment with leucine aminopeptidase^a

Group	Ileum	Cecum	Colon	Composite
Treated	1.00 ± 0.21	0.33 ± 0.17	0.60 ± 0.22	1.90 ± 0.43*
Not treated	1.33 ± 0.17	0.75 ± 0.16	1.22 ± 0.22	3.22 ± 0.36

^a Rat pups were dosed twice daily from days 7 to 11 with 0.48 mg of LAP in 200 µl of PBS, and orally challenged with 10³ *C. parvum* oocysts at 9 days of age.

* Significantly ($P < 0.05$) less than the score for rats not treated.

Table 2, histology scores were lower in treated than in control rats for all three tissues examined. The composite score for treated rats was significantly ($P < 0.05$) lower than the composite score for control rats.

These data confirm previous results (Akili and Harp, 2000; Harp, 2003) indicating that a substance is present in the intestinal mucosa of adult cows that can transfer protection against *C. parvum* infection to infant rats. The present data suggest the activity is associated with an extrinsic membrane protein. Furthermore, a protein band with an apparent molecular mass of 54 kDa is present in protective, but not non-protective intestinal mucosal preparations. A major protein species in this band has homology to LAP, and a commercial preparation of LAP was able to confer some protection from *C. parvum* infection in infant rats. These data suggest the possibility that the protective protein in the adult mucosal is LAP. While hexameric LAP from bovine lens has a molecular weight of 326 kDa, the individual subunits have a molecular weight of 54 kDa (Cuypers et al., 1982). Thus under the denaturing and reducing conditions of SDS-PAGE, the subunits may have dissociated and migrated at 54 kDa. A second possibility is that the protective protein is not LAP, but rather a different protein with high homology at the N-terminus to LAP. In this case the degree of homology may have been sufficient for LAP to confer some protection from *C. parvum* infection in our assay. The preliminary data in the present study are not sufficient to distinguish between these possibilities. In either case, one might speculate that the protective effect of the protein may be mediated by binding to, or blocking binding to, a receptor critically involved in *C. parvum* infection.

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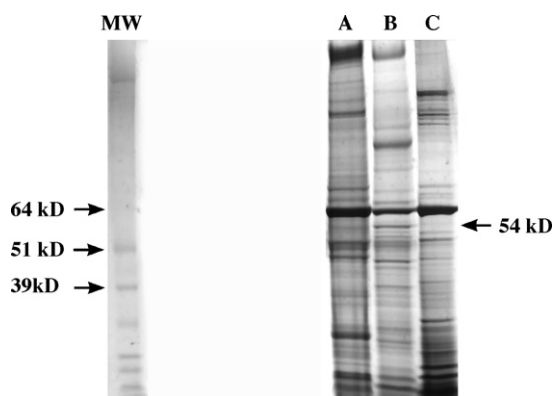


Fig. 1. Silver stained SDS-PAGE of extrinsic membrane fractions from (A) cow mucosa, (B) adult rat mucosa, (C) calf mucosa. MW: molecular weight standards. Note band present at 54 kDa in cow and adult rat preparations, but not in calf preparation. Intervening lanes showing crude membrane and pellet fractions originally between MW standards and ABC were digitally removed. Original alignment of bands between lanes is unaltered.

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